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2. Patent application number *(The Patent Office will fill in this part)* **9928323.6**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

CYCLACEL LIMITED,
MARQUIS HOUSE,
67-68 JERMYN STREET,
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Patents ADP number *(if you know it)*

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4. Title of the invention — PEPTIDES

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PEPTIDES

The present invention relates to substances and their therapeutic use, and in particular to specific regions of $p21^{WAF1}$ that bind to G1 and S phase specific cyclins, preferably ones activating cdk2 and to substances and mimetics based on this region. The invention also relates to assay methods and means for identifying substances useful for interfering with p21/Cdk/cyclin interaction, and preferably inhibiting Cdk2 activity.

$p21^{WAF1}$ is an inhibitor of both the G1 cyclin dependent protein kinases (CDKs; which control the progression from G1 into S phase) (Harper et al., 1995) and proliferating cell nuclear antigen (PCNA; an essential DNA-replication factor) (Florez-Rozas et al., 1994; Waga et al., 1994). Thus, inhibition of the function of either CDKs or PCNA provides, in theory, two distinct avenues for development of drug discovery programmes which are based on the activity of $p21^{WAF1}$. The PCNA binding function of $p21^{WAF1}$ can be mimicked by a 20-amino acid peptide derived from the C-terminal domain of $p21^{WAF1}$ and this peptide is sufficient to partially inhibit SV40 replicaton in vitro (Warbrick et al., 1995).

Despite its PCNA binding role, the primary function of the $p21^{WAF1}$ protein as a growth suppressor appears to be inhibition of the G1 cyclin-CDK complexes (Chen et al., 1995; Harper et al., 1995; Luo et al., 1995; Nakanishi et al., 1995b). Luo et al. (1995) reported the N-terminal domain of p21, composed of residues 1-75, to act as a CDK-inhibitor in vitro, inhibiting cyclin E-Cdk2.

WO 97/42222 (Cyclacel Ltd) discloses peptide fragments of $p21^{WAF1}$ that interact with cdk4/cyclin D1. Thus it was observed that $p21_{(16-35)}$ and $p21_{(46-65)}$ bind to cdk4 and cyclin D1 respectively. Of these, only $p21_{(16-35)}$ was observed to inhibit cdk activity. $p21_{(141-160)}$ was observed to bind to cdk4 and cyclin D1 and to be a potent inhibitor of cdk4.

This data supported the known phenomena of peptides including the sequence LFG as being the binding motif essential for the interaction of the p21 family with cyclins [Chen J et al., Lin J et al. and Russo AA et al.] and the further known properties of the amino-terminal half of p21 as being required for binding to cdk complex.

The sequence $p21_{(141-160)}$ (disclosed in WO97/42222 and Ball K. et al) was subjected to analysis in order to determine the minimum length of an inhibitory peptide upon which novel antiproliferative drugs could be designed. This led to the identification of an inhibitory motif comprising RRLIF ($p21_{(155-159)}$), the bold residues being described as essential for activity and the underlined residue contributing towards inhibitory activity. Further observations in these disclosures include the retention of inhibitory activity against cyclin D1-cdk4 by the peptide KRRLIFSK ($p21_{(152-163)}$) and that the substitution of aspartic acid at position 149 by alanine surprisingly reduced the IC_{50} of the full length peptide from 100 nM to 46 nM.

In summary, WO97/42222 and Ball et al teach that there are sequences within the carboxy terminal region of p21 that are capable of interacting with cdk4/cyclin D in a manner that is inhibitory to cdk4 and further involves specific binding to cyclin D. Though the peptide $p21_{(141-160)}$ is described as being preferred, an 8-mer comprising $p21_{(154-163)}$ was inhibitory, but at higher concentrations. Finally, alanine replacement at position 149 within $p21_{141-160}$ increased the inhibitory activity. Thus, although the art indicates that this is an interesting region of p21 to investigate, no guidance is provided as to the identity of further fragments that would be preferably active against cdk4/cyclin D or any other cdk enzymes.

Adams DA et al. (Mol Cell Biol (1996) 16(12) 6623-6633) discloses N- and C-terminal regions of p21 that putatively bind to cdk2/cyclin. A 14-mer (p21 149-162) is disclosed as inhibiting the binding of cyclin A to E2F1 and the binding of cyclins A and E to GST-p21. An amino acid sequence containing 8 amino acids derived from the transcription factor E2F1 was shown to bind to cyclin A/E-cdk2 complexes. An alanine scan of the 8-mer identified, on a qualitative level that certain modified forms of the peptide retained this activity.

An aim of the present invention has been to identify further peptides derived from p21 that retain or improve upon the inhibitory activities described in the art, particularly with regard to substrate specificity and peptide chain length.

A first aspect of the present invention therefore relates to a p21 derived peptide of formula;

DFYHSKRRLIF (SEQ ID No. 1)

or such a peptide

(i) bearing a further amino acid residue at either end; or,

(ii) having upto 5 amino acid residues deleted from the N-terminal end;

and variants thereof wherein at least one amino acid residue is replaced by an alternative natural or unnatural replacement amino acid residue, with the proviso that the motif RXLXF is retained. The peptide of SEQ ID No. 1 corresponds to p21(149-159).

A second aspect of the present invention relates to a p21 derived peptide of formula (I);

$$X_1X_2X_3RX_4LX_5F \qquad (I)$$

wherein X_1, X_3, X_4 and X_5 are any amino acid and X_2 is serine or alanine; and variants thereof.

Although the peptides of the first aspect and in some embodiments of the second aspect, include the described cdk4-inhibitory motif RRLIF, the peptides of the present invention have been shown to display preferential selectivity for cdk2 over cdk4 in contrast to those described in Ball et al.(supra). Further advantages of the above peptides relate to their specificity, particularly for G1 control cdk's, such as cdk2/cyclinE, as opposed to mitotic control enzymes including cdk's such as cdk1/cyclin B or A and protein kinase Cα (PKCα).

Further evidence of the unexpected observation that these peptides display activity against cdk4 and cdk2 is that Ball et al. described how N-terminal truncation of $p21_{140-160}$ reduced activity. The disclosure therein of RRLIF as being the cdk4-inhibitory motif was made on a theoretical basis rather than a demonstration that a peptide of that size would retain inhibitory activity. Thus, the present invention has demonstrated, in contrast to the information available in the art, that shorter, in some cases more specific and/or potent inhibitors of cyclin-cdk, especially cyclin E/cdk2 interaction may derived from within the sequence p21 (141-160).

one embodiment of the first aspect of the invention, the peptide may include a further amino acid residue at either the N- or C-terminus. The further residue is preferably selected from the polar residues C, N, Q, S, T and Y, and is preferably threonine when added to the N-terminus and serine, when added to the C-terminus. These last recited preferred embodiments correspond to the sequences 148-159 and 149-160 of p21 respectively. In an alternative embodiment, upto 5 amino acid residues may be deleted from the N-terminal end of SEQ ID No. 1. Such truncation may therefore give rise to peptides corresponding to p21(150-159), p21(151-159), p21(152-159), p21(153-159) and p21(154-159) or wherein an additional serine residue is added to the C-terminal end to p21(150-160), p21(151-160), p21(152-160), p21(153-160) and p21(154-160). Preferably, from 2 to 4 residues are deleted, most preferably three are deleted. In each of these preferred embodiments it is preferable that, when present the serine residue corresponding to p21(153) is replaced by an alanine residue.

Considering the second aspect of the invention, X_2 is preferably alanine as this provides a significant increase in the efficacy of the peptide and X_5 is preferably a non-polar amino acid residue, more preferably isoleucine or glycine, most preferably isoleucine. Of the remaining groups, X_1, X_3 and X_4, X_1 and X_4 are both preferably basic amino acid residue, X_1 is more preferably histidine and X_4 more preferably arginine. X_3 may be a basic or polar residue, preferably lysine or cysteine. A preferred peptide in accordance with the second aspect is that of SEQ ID No. 2;

HX_2KRRLX_5F (SEQ ID No. 2)

wherein X_2 and X_5 have the same meanings and preferences as above. When X_2 is serine and X_5 isoleucine the peptide corresponds to the sequence 152-159 of p21 and may hereinafter be referred to as p21(152-159). A further aspect of the invention therefore relates to a peptide HX_2KRRLX_5F (SEQ ID No. 2) and variants thereof, especially, wherein at least one amino acid residue is replaced by an alternative natural or unnatural replacement amino acid residue.

As used herein the term "variant" is used to include the peptides of SEQ ID Nos 1 and 2 being modified by at least one of; deletion, addition or substitution of one or more amino acid residues, or by substitution of one or more natural amino acid residues by the

rresponding D-stereomer or by a non-natural amino acid residue, chemical derivatives of the peptides, cyclic peptides derived from the peptides or from the peptide derivatives, dual peptides, multimers of the peptides and any of said peptides in the D-stereomer form or the order of the final two residues at the C-terminal residues are reversed, provided that such variants retain the activity of the parent peptide. As used hereinafter, the term "substitution" is used as to mean "replacement" i.e. substitution of an amino acid residue means its replacement.

Preferably, the variants involve the replacement of an amino acid residue by one or more, preferable one of those selected from the residues of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

Such variants may arise from homologous substitution i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine, diaminobutyric acid, norleucine, pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

As used herein, amino acids are classified according to the following classes;

basic; H, K, R
acidic; D, E
non-polar; A, F, G, I, L, M, P, V, W
polar; C, N, Q, S, T, Y,
(using the internationally accepted amino acid single letter codes)
and homologous and non-homologous substitution is defined using these classes. Thus, homologous substitution is used to refer to substitution from within the same class, whereas non-homologous substitution refers to substitution from a different class or by an unnatural amino acid.

The variants may also arise from replacement of an amino acid residue by an unnatural amino acid residue that may be homologous or non-homologous with that it is replacing.

such unnatural amino acid residues may be selected from;—alpha* and alpha-disubstituted* amino acids, N-alkyl amino acids*, lactic acid*, halide derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine*, p-I-phenylalanine*, L-allyl-glycine*, ß-alanine*, L-α-amino butyric acid*, L-γ-amino butyric acid*, L-α-amino isobutyric acid*, L-ε-amino caproic acid#, 7-amino heptanoic acid*, L-methionine sulfone#*, L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline#, L-thioproline*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, L-Phe (4-amino)#, L-Tyr (methyl)*, L-Phe (4-isopropyl)*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, L-diaminopropionic acid # and L-Phe (4-benzyl)*. The notation * has been utilised for the purpose of the discussion above, to indicate the hydrophobic nature of the derivative whereas # has been utilised to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

With particular reference to the first aspect of the invention (SEQ ID No. 1), a variant peptide may involve the replacement of an amino acid residue by an alanine residue. In the first aspect of the present invention, such substitution preferably takes place at any of positions 150, 151, 152, 153, 154, 158 or 160 which all display a greater selectivity for cdk2/cyclin E inhibition than cdk4/cyclin D1 inhibition as described below. Most preferably, such alanine replacement occurs at position 153 where in addition to an increase in selectivity, the observed IC_{50} is at least two orders of magnitude greater that for the corresponding parent peptide ($p21_{149-160}$). In respect of the second aspect of the invention, it is also preferable that amino acid replacement is by an alanine residue, most preferably at the 153 position. Furthermore, in respect of this aspect of the invention, the variant may include the deletion of the N-terminal asparagine residue resulting in the peptide corresponding to p21(150-159). Most preferably the peptide is D F Y H A K R R L I F S.

With particular reference to the second aspect of the invention (formula (I), a variant peptide may additionally involve the replacement of an amino acid residue by an alanine residue, the deletion of X_1 or the reversal of X_5 and the terminal phenylalanine residue. These options are also applicable to the peptide SEQ ID No. 2 which may therefore, by way of example result in the peptides X_2KRRLX_5F and $HX_2KRRLFX_5$. Most preferably, the peptide is H A K R R L I F. Further variants include the addition of up to 4

mino acid residues at the C- or N-terminus being SKRK or FYHS respectively.

Considering the peptide HAKRRLIF (1-8), further suitable variants may include any one of or optionally at least one of the following;

(a) replacing the histidine (1) with a natural or unnatural amino acid residue providing an identical balance of hydrogen bonding/polar interaction, such as 3-pyridylalanine (Pya), 2-thienylalanine (Thi), homoserine (Hse), phenylalanine, or diaminobutyric acid (Dab),

(b) replacement of alanine (2) with alternative a natural or unnatural amino acid residue having a slightly larger aromatic or aliphatic side chain, such as glycine, aminobutyric acid (Abu), norvaline (Nva), t-butylglycine(Bug), valine, isoleucine, phenylglycine (Phg) or phenylalanine,

(c) replacement of lysine (3) with an uncharged natural or unnatural amino acid residue, such as norleucine (Nle), aminobutyric acid (Abu) or leucine

(d) replacement of arginine (4) by an uncharged natural or unnatural amino acid residue, such as citrulline (Cit), homoserine, histidine, norleucine (Nle) or glutamine,

(e) replacement of arginine (5) by an uncharged hydrogen bond donor or acceptor natural or unnatural amino acid residue, such as asparagine, proline, serine, aminoisobutyric acid ((Aib) or sarcosine (Sar),

(f) replacement of lysine (6) or isoleucine (7) with alternative a natural or unnatural amino acid residue having a slightly larger aromatic or aliphatic side chain, such as norleucine, norvaline, cyclohexylalanine (Cha), phenylalanine or 1-naphthylalanine (1Nal),

(g) replacing the phenylalanine (8) with a natural or unnatural amino acid residue of higher steric bulk such as leucine, cyclohexylalanine (Cha), homophenylalanine (Hof), tyrosine, para-fluorophenylalanine (pFPhe), meta-fluorophenylalanine (mFPhe), trptophan, 1-naphthylalanine (1Nal), 2-naphthylalanine (2Nal), biphenylalanine(Bip) or (Tic).

The three letter notations appearing above are used in Figure 4 which provides examples of these further variants.

Thus, in accordance with the first embodiment of the invention, the peptide may be

lected from;

DFYHAKRRLIFS
TDFYHSKRRLIF,
AFYHSKRRLIFS,
DAYHSKRRLIFS,
DFAHSKRRLIFS,
DFYASKRRLIFS,
DFYHAKRRLIFS,
DFYHSARRLIFS,
DFYHSKRALIFS,
DFYHSKRRLAFS,
DFYHSKRRLIFA,
FYHSKRRLIFS,
YHSKRRLIFS,
HSKRRLIFS,
DFYHSKRRLIF,
FYHSKRRLIF
YHSKRRLIF
HSKRRLIF,
SKRRLIF,
KRRLIF.

In accordance with the second embodiment of the invention, the peptide may be selected from;

HSKRRLIF,
HAKRRLIF,
HSKRRLFG,
HAKRRLFG,
KACRRLFG,
KACRRLIF.

A further embodiment of the present invention relates to assays for candidate substances that interact with cyclin interaction with cdk's, especially cdk2 and cdk4. Such assays are

sed upon the observation that the peptides of the invention have been shown to bind to cyclin despite not including the generally considered "cyclin binding motif" as discussed above. Furthermore, it has been shown that the peptides of the second and further aspects of the invention competitively inhibit the binding of a peptide of the first aspect of the invention. Thus, such assays may involve incubating a candidate substance with a cyclin and a peptide of the invention and detecting either the candidate-cyclin complex or free (unbound) peptide of the invention. An example of the latter would involve the peptide of the invention being labelled such as to emit a signal when bound to a cdk. The reduction in said signal being indicative of the candidate substance binding to, or inhibiting peptide-cyclin interaction.

Suitable candidate substances include peptides, especially of from about 5 to 30 or 10 to 25 amino acids in size, based on the sequence of the various domains of p21, or variants of such peptides in which one or more residues have been substituted. Peptides from panels of peptides comprising random sequences or sequences which have been varied consistently to provide a maximally diverse panel of peptides may be used.

Suitable candidate substances also include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grafted antibodies) which are specific for p21 or cyclin binding regions thereof. Furthermore, combinatorial libraries, peptide and peptide mimetics, defined chemical entities, oligonucleotides, and natural product libraries may be screened for activity as inhibitors of cyclin-cdk interaction in assays such as those described below. The candidate substances may be used in an initial screen in batches of, for example 10 substances per reaction, and the substances of those batches which show inhibition tested individually. Candidate substances which show activity in *in vitro* screens such as those described below can then be tested in whole cell systems, such as mammalian cells.

The peptides of the invention and substances identified or identifiable by the assay methods of the invention may preferably be combined with various components to produce compositions of the invention. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition of the invention

ay be administered by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each protein may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

Pharmaceutically acceptable salts of the peptides of the invention include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

Examples

Experimental Procedures

Peptide synthesis. Peptides were assembled using a Multipin Peptide Synthesis Kit (Chiron Technologies, Clayton, VIC, Australia) (Valerio et al., 1993). Standard solid-phase chemistry based on the Fmoc protecting group was employed (Fields et al., 1990). Peptides were side-chain deprotected and cleaved from the synthesis support using methods as described (King et al., 1990). All peptides were purified by preparative reversed-phase HPLC or solid phase extraction, isolated by lyophilisation, and were analyzed by analytical HPLC and mass spectrometry (Dynamo DE MALDI-TOF spectrometer, ThermoBioAnalysis).

Proteins:

PKCα - 6 x His, CDK4 - 6 x His, CDK2-6 x His/Cyclin E-6 x His, CDK1-6 x His/Cyclin B-6 x His – His – tagged CDK2/Cyclin E and CDK1/Cyclin B were co-expressed and PKCα,and CDK4 were singularly expressed in Sf 9 insect cells infected with the appropriate baculovirus constructs. The cells were harvested two days after infection by low speed centrifugation and the proteins were purified from the insect cell pellets by Metal-chelate chromatography. Briefly, the insect cell pellet was lysed in Buffer A (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NP40 and 5 mM β-marcaptoethanol, 1 mM NaF. 1 mM Na3VO4 and Protease Inhibitors Coctail (Sigma) containing AEBSF, pepstatin A, E 64, bestatin, leupeptin) by sonication. The soluble fraction was cleared by centrifugation and loaded onto Ni-NTA-Agarose (Quiagen). Non bound proteins were washed off with 300 mM NaCl, 5-15 mM Imidazole in Buffer A and the bound proteins were eluted with 250 mM Imidazole in Buffer A. The purified proteins were extensively dialyzed against Storage buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 2 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.02% NP40, 10% v/v Glycerol) aliquoted and stored at -70°C.

PKC-α - 6 x His was purified the same way but using different buffers- 50 mM NaH2PO4, pH 8.0 and 0.05% Triton X-100 instead of Tris and NP40 respectively.

Cyclin D1 and p21 were expressed in E coli BL21 (DE3) using PET expression vectors. BL21 (DE3) was grown at 37°C with shaking (200 rpm) to mid-log phase (OD600

n=0.6). Expression was induced by the addition of IPTG at a final concentration of 1 mM, and the culture was incubated for a further 3h. The bacteria were than harvested by centrifugation, and the cell pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 10% sucrose. Both proteins were purified from inclusion bodes. Briefly, the bacterial cells were lysed by treatment with lysosyme and sonication. The insoluble fraction was pelleted by centrifugation. The inclusion bodies were purified by repetitive washing of the insoluble fraction with 50 mM Tris-Hcl pH 8.0, 2 mM EDTA, 100 mM NaCl and 0.5% Triton X-100. Purified inclusion bodies were solubilized with the same buffer, containing 6M urea. The proteins were refolded by slow dilution with 25 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM DTT, 1 mM EDTA, 0.2% NP40. After concentration by ultrafiltration (Amicon concentration unit) the purified proteins were aliquoted and stored at –70°C.

GST-Rb – An E coli expression construct containing the hyperphosphorylation domain of pRb (amino acids 773-924) was purified on a Glutathione-Sepharose column according to the manufacturers instructions (Pharmacia). For the 96-well format "in vitro" kinase assay GST-Rb was used immobilized on Glutathione-Sepharose beads.

Enzyme Assays

CDK4/Cyclin D1, CDK2/Cyclin E, CDIK1/Cyclin B kinase assays

Phosphorylation of GST-Rb

GST-Rb phosphorylation, induced by CDK4/Cyclin D1, CDK2/Cyclin E or CDK1/Cyclin B was determined by incorporation of radio-labeled phosphate in GST-Rb using radiolabelled ATP in 96-well format *in vitro* kinase assay. The phosphorylation reaction mixture (total volume 40 µl) consisted of 50 mM HEPES pH 7.4, 20 mM MgCl2, 5 mM EGTA, 2 mM DTT, 20 mM β-glycerophosphate, 2 mM NaF, 1 mM Na3VO4, Protease Inhibitors Cocktail (Sigma, see above), BSA 0.5mg/ml, 1 µg purified enzyme complex, 10 µl of GST-Rb-Sepharose beads, 100 µM ATP, 0.2µCi ^{32}P-ATP. The reaction was carried out for 30 min at 30°C at constant shaking. At the end of this period 100 µl of 50 mM HEPES, pH 7.4 and 1 mM ATP were added to each well and the total volume was transferred onto GFC filtered plate. The plate was washed 5 times with 200 100 µl of 50 mM HEPES, pH 7.4 and 1 mM ATP. To each well were added 50 µl scintillant liquid and the radioactivity of the samples was measured on Scintilation counter (Topcount, HP). The IC50 values of different peptides were calculated using GraFit software.

Phosphorylation of Histone

Histone 1 phosphorylation induced by CDK2/Cyclin E and CDK1/Cyclin B was measured using similar method. The concentration of Histone 1 in the kinase reaction was 1mg/ml (unless different stated). The kinase reaction was stopped by 75 mM Phosphoric acid (100 µl per well) and the reaction mixture was transferred onto P81 plates. The plates were washed 3 times with 200 µl 75 mM Phosphoric acid.

Protein Kinase C (PKC) α Assay

PKCα kinase activity was measured by the incorporation of radio-labeled phosphate in Histone 3. The reaction mixture (total volume 65 µl) consist of 50 mM Tris-HCl, 1 mM Calcium acetate, 3 mM DTT, 0.03 mg/ml Phosphatidilserine, 2.4 µg/ml PMA, 0.04% NP40, 12 mM Mg/Cl, purified PKCα -100 ng, Histone 0.2mg/ml, 100 µM ATP, 0.2 µCi [γ-^{32}P]-ATP. The reaction was carried over 15 min at 37°C in microplate shaker and was stopped by adding 10 µl 75 mM Phosphoric acid and placing the plate on ice. 50 µl of the reaction mixture was transferred onto P81 filterplate and after washing off the free radioactive phosphate (3 times with 200 µl 75 mM Phosphoric acid per well) 50 µl of scintilation liquid (Microscint 40) were added to each well and the radioactivity was measured on Scintilation counter (Topcount, HP).

ERK-2 (MAP Kinase) Assay

ERK-2 kinase activity was measured by the incorporation of radio-labeled phosphate in Myelin Basic Protein (MBP), catalyzed by purified mouse ERK2 (Upstate Biotecnoligies). The reaction mixture (total volume 50 µl) consisted of 20 mM MOPS, pH 7.0, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 10 mM MgCl, 100 µM ATP, 0.2 µCi [γ-^{32}P]-ATP.

Competitive Cyclin D1/Cyclin A binding assay (ELISA).

Biotinylated p21 (149-159) – DFYHSKRRLIF was immobilized on Streptavidin coated 96-well plates (PIERCE). Different amounts of a competitor peptide were mixed with Cyclin D1/Cyclin A and than loaded onto the plate with immobilized biotinylated p21 (149-159). The amount of bound Cyclin D1/Cyclin A was immunodetected and quantified by Turbo-ELISA reagent (PIERCE). The IC 50 values (a concentration of the competitor

peptide which inhibits 50 % of Biotin-p21 (149-159) – Cyclin D1/Cyclin A binding) were calculated using GraFit software.

Results

Structure-Activity Relationships (SAR) of p21 (149-160) in CDK4/Cyclin D1 kinase assay.

Previous work of K. Ball et al., (1995) showed that a 20 amino acid (AA) peptide, derived from the C-terminus of p21- KRRQTSATDFYHSKRRLIFS – p21 (141-160) binds to CDK4 and Cyclin D1 and is able to inhibit in vitro kinase activity of CDK4-Cyclin D1 complex.

We have demonstrated that a shorter sequence being a 12 amino acid peptide DFYHSKRRLIFS – p21 (149-160) appeared to have very similar activity as the original 20-mer peptide of Ball et al.. with respect to in vitro inhibitory activity in vitro CDK4-Cyclin D1 kinase.

A detailed SAR analysis of p21 (149-160) was done in 96-well format CDK4-Cyclin D1 kinase assay using different peptide derivatives – truncations and alanine substitutions. The results are shown in Table 1.

SAR of p21 (149-160) in CDK2/Cyclin E kinase assay.

P21 (141-160) peptide was shown to inhibit CDK2-Cyclin E induced phosphorylation of GST-Rb (Ball et al., 1995) at concentration 40 times its IC50 of cdk4/cyclin D1. The results herein show that a truncated form– p21 (149-160) and variants thereof, retain very good potency to inhibit the CDK2-Cyclin E induced phosphorylation of GST-Rb and in many cases the peptides were shown to be preferentially inhibitory of cdk2 as opposed to cdk4. Detailed SAR of p21 (149-160) were determined in CDK2-Cyclin E *in vitro* kinase assay. The data are shown in Table 1.

A comparison between the SAR of p21 (149-160) in CDK2-Cyclin E and CDK4-Cyclin

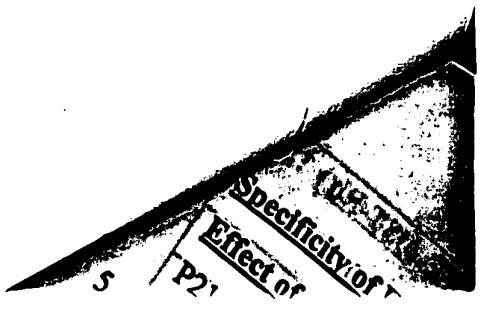
l kinase assays shows a higher inhibitory activity towards CDK2-Cyclin E than to CDK4-Cyclin D1. Alanine mutation of Ser153 increases 100 fold the potency of the peptide to inhibit the CDK2-Cyclin E but has little effect on CDK4-Cyclin D1 induced phosphorylation of GST-Rb. For both inhibitory activities of p21 (149-160) the most important residues are Arg155, Leu 157 and Phe 159. The CDK4-Cyclin D1 inhibitory activity of p21 (149-160) tolerates less changes than the CDK2-Cyclin E one.

Using identical assays, the sequence p21(148-159) was shown to be active against both cdk2/cyclin E and cdk4/cyclin D1.

Table 1. SAR of p21 (149-160) in CDK4-cyclin D1 and CDK2-Cyclin E kinase assay

Peptide	Sequence	CDK2/E IC50 [μM]	CDK2/E % Inhibition	CDK4/D1 IC50 [μM]	CDK4/D1 % Inhibition
P21 (148-159)	TDFYHSKRRLIF	2.2 ± 0.4	85	15 ± 3	72
P21 (149-160)	DFYHSKRRLIFS	4.5 ± 0.5	80	20 ± 2	70
P21 (150-161)	FYHSKRRLIFSK	26 ± 6.2	70	41 ± 10	70
P21 (151-162)	YHSKRRLIFSKR	17.6 ±6.9	80	45 ± 10	60
P21 (152-163)	HSKRRLIFSKRK	8.7 ± 2.5	90	34 ± 6	80
P21 (153-164)	SKRRLIFSKRKP	46 ± 33	70	-	40
P21 (149-160)149A	AFYHSKRRLIFS	11 ± 2	70	22 ± 4	72
P21 (149-160)150A	DAYHSKRRLIFS	5.9 ± 0.4	85	37 ± 6	76
P21 (149-160)151A	DFAHSKRRLIFS	5.3 ± 0.6	80	121 ± 31	56
P21 (149-160)152A	DFYASKRRLIFS	5.1 ± 0.5	80	73 ± 42	60
P21 (149-160)153A	DFYHAKRRLIFS	0.041 ± 0.0049	80	10	52
P21 (149-160)154A	DFYHSARRLIFS	12.9 ±2.4	80	200	50

Peptide	Sequence	CDK2/E IC50 [μM]	CDK2/E % Inhibition	CDK4/D1 IC50 [μM]	CDK4/D1 % Inhibition
P21 (149-160)155A	DFYHSKARLIFS	-	25	-	30
P21 (149-160)156A	DFYHSKRALIFS	30 ± 8	70	-	30
P21 (149-160)157A	DFYHSKRRAIFS	-	30	-	30
P21 (149-160)158A	DFYHSKRRLAFS	14 ± 3	80	53 ± 20	61
P21 (149-160)159A	DFYHSKRRLIAS	-	20	-	35
P21 (149-160)160A	DFYHSKRRLIFA	5.4 ± 1.1	70	40	60
P21 (150-160)	FYHSKRRLIFS	6.8 ± 1.0	80	22 ± 5	70
P21 (151-160)	YHSKRRLIFS	7.3 ± 0.8	80	20 ± 1	70
P21 (152-160)	HSKRRLIFS	3.4 ± 0.2	80	32 ± 6	65
P21 (149-159)	DFYHSKRRLIF	2 ± 0.2	75	13 ± 2	70
P21 (150-159)	FYHSKRRLIF	5.8 ± 1	80	19 ± 3	70
P21 (151-159)	YHSKRRLIF	7 ± 2	80	16 ± 1	70
P21 (152-159)	HSKRRLIF	3.4 ± 1	80	21 ± 4	72
P21 (153-159)	SKRRLIF	7.7 ± 0.5	80	54	72
P21 (154-159)	KRRLIF	11 ± 1.3	80	>200	72



Specificity of Enzyme inhibition

Effect of p21 (149-160) on CDK2-Cyclin E induced phosphorylation of Histone 1.

P21 (149-160) was tested for inhibitory activity in CDK2-Cyclin E kinase assay with Histone 1 as a substrate. The peptide was completely inactive as an inhibitor of CDK2-Cyclin E induced phosphorylation of Histone 1 (Figure 1).

One possible mechanism for inhibitory action is competition of the peptide with the substrate for binding to the kinase complex. If this is so, the peptide inhibitory activity will depend on the substrate concentration. We determined the IC 50 of p21 (149-160) in presence of different concentrations of Histone 1 – 0.1, 0.2, 0.7 and 1 mg/ml. P21 (149-160) did not inhibit CDK2-Cyclin E induced phosphorylation of Histone 1 at any of the substrate concentrations used.

Similarly, p21 (149-160)153A was also tested for its ability to inhibit Histone 1 phosphorylation induced by the same kinase complex (Figure 2). Even this very strong inhibitor of the GST-Rb phosphorylation was completely inactive in inhibition of the phosphorylation of Histone 1 induced by CDK2-Cyclin E kinase complex.

Effect of p21 (149-160) and its derivatives on CDK1-Cyclin B kinase activity.

P21 (149-160) and its derivatives were tested for an effect on CDK1-Cyclin B kinase activity to phosphorylate Histone 1 or GST-Rb (Table 2). P21 (149-160) and its alanine mutant – p21 (149-160)153A did not have any significant effect on the CDK1-Cyclin B induced phosphorylation of Histone1. None of the tested peptides was able to inhibit significantly the CDK1-Cyclin B induced phosphorylation of GST-Rb either. Only the highest peptide concentrations used by us (200 μM) had slight inhibitory effect on CDK1-Cyclin B kinase activity.

Table 2 Inhibition of CDK1-Cyclin B induced phosphorylation of Histone 1 and GST-Rb by p21 derived peptides.

Peptide	Sequence	Histone IC50 [μM]	GST-Rb IC50 [μM]
P21 (149-160)	DFYHSKRRLIFS	> 200	200
P21 (149-160)153A	DFYHAKRRLIFS	200	>200
P21 (149-159)	DFYHSKRRLIF	Not tested	>200

Effect of purified P21^{WAF1} on CDK4-Cyclin D1 and CDK2-Cyclin E kinase activity

In order to evaluate the selectivity, specificity and potency of p21 (149-160) and its derivatives we compared their effect with the one of purified p21 on kinase activity of CDK2-Cyclin E and CDK4-Cyclin D1. The IC 50 values characterizing the inhibition of CDK4-Cyclin D1 and CDK2-Cyclin E induced phosphorylation of GST-Rb and CDK2-Cyclin E induced phosphorylation of Histone1 by purified p21 ^{WAF1} are shown in Table 3. The IC 50 of the most active peptide – p21 (149-160) 153A for CDK2-Cyclin E induced phosphorylation of GST-Rb was 40 nM which is approximately 50 fold higher than the IC 50 value for p21^{WAF1}. Purified p21 though, inhibited strongly the CDK2-Cyclin E induced phosphorylation of GST-Rb as well as Histone 1. The peptides derived from p21 ^{WAF1} - p21 (149-160) and p21 (149-160)153A peptides specifically inhibit the GST-Rb phosphorylation, but do not inhibit the Histone 1 phosphorylation induced by CDK2-Cyclin E. This substrate specific effect of p21 (149-160) and its derivatives strongly suggest a mechanism of competitive binding of the peptide inhibitors and Rb to CDK2-Cyclin E or CDK4-Cyclin D1. The fact that p21 (149-160) and its derivatives did not inhibit significantly the CDK1-Cyclin B induced phosphorylation of GST- Rb excludes a possibility for direct binding of the peptide to the substrate (see Table 2).

Table 3 Inhibition of CDK4-Cyclin D1 and CDK2-Cyclin E kinase activity by purified p21^{WAF1}

Kinase complex	Substrate	Inhibition by p21 ^{WAF1} IC50 [nM]
CDK4-Cyclin D1	GST-Rb	6.5 ± 0.8
CDK2-Cyclin E	GST-Rb	0.7 ± 0.2
CDK2-Cyclin E	Histone 1	1.8 ± 0.4

GST-Rb IC50 [μM]

21 (149-160) and its derivatives do not inhibit PKCα and ERK2 kinase activity *in – vitro*

To investigate further the specificity of p21 (149-160) and its derivatives we investigated the effect of the strongest inhibitors of CDK2-Cyclin E and CDK4-Cyclin D1 complexes on PKC α and ERK2 kinase activity (Table 4). None of the tested peptides (at concentrations up to 100 μM) had any inhibitory effect on PKCα phosphorylation of Histone 3 or ERK2 phosphorylation of Myelin Basic Protein. These results demonstrate further the selectivity of the inhibitory effect of the peptides derived from p21 C-terminus.

Table 4 Effect of p21 derived peptides on PKCα and ERK2 kinase activity. For a comparison the peptide inhibitory activity towards CDK2-Cyclin E and CDK4-Cyclin D1 is given as well.

Peptide	Sequence	CDK4-D1 IC50 [μM]	CDK2-E IC50 [μM]	PKCα IC50 [μM]	ERK2 IC50 [μM]
P21 (148-159)	T D F Y H S K R R L I F	15	2.2	-	-
P21 (149-160)	D F Y H S K R R L I F S	20	4.5	-	-
P21 (149-160) S153A	D F Y H A K R R L I F S	10	0.04	-	-
P21 (149-159)	D F Y H S K R R L I F	13	2	-	-
P21 (150-159)	F Y H S K R R L I F	19	5.8	-	-
P21 (151-159)	Y H S K R R L I F	16	7	-	-
P21 (152-159)	H S K R R L I F	21	3.4	-	-

P21 (149-159) binds to the Cyclin, but does not bind to the CDK sub-unit of CDK/Cyclin complex. Binding of the peptide to the Cyclin does not disrupt the complex.

Biotinylated version of p21 (149-159) – DFYHSKRRLIF was used in "pull down" experiments with purified CDK2, CDK4, Cyclin D1, Cyclin A, CDK2/Cyclin A or CDK4/Cyclin D1 kinase complex to determine the binding partner of the peptide. The

Biotinylated peptide was pre-immobilized on Streptavidin – agarose beads (see Materials and Methods for details). On Figure 3 are shown the profiles of the "pulled down" proteins, after SDS-PAGE , Western blotting and immunodetection. P21 (149-159) binds to Cyclin A and Cyclin D1, but failed to "pull down" any CDK2 or CDK4 in the absence of their cyclin partners. CDK2 and CDK4 were "pull down" though with biotinylated p21 (149-159) – Streptavidin – agarose beads when they were in a complex with Cyclin A or Cyclin D1 respectively. This result suggest that binding of the peptide to the Cyclin sub-unit does not disrupt the CDK/Cyclin complex. Such a method may be utilised either alone or together with a candidate substance to identify cyclin binding moities and/or inhibitors of cyclin-cdk interaction.

Comparison between peptides, containing ZRXL substrate recognition motif.

Adams et al., (1996) identified a motif – **ZXRL** which is present in many CDK2/Cyclin A (E) substrates -E2F family transcription factors and pRb family proteins; the same motif is present in p21 (N- and C –terminus), p27 and p57 kinase inhibitors (see Fig 2 in Adams et al.). When the substrate recognition motif was mutated in p107 (Rb related protein) or E2F1 their phosphorylation by CDK2-Cyclin A was prevented (Adams et al., 1996).

Our p21 (149-160) SAR data clearly show though that two amino acids outside of **ZXRL** motif are very important for the kinase inhibitory activity of p21 (C-terminus) derived peptide – A153 (which increases the potency approximately 100 fold) and F159 (which is vital for the kinase inhibition). To evaluate the importance of these flanking the ZXRL motif regions we designed peptides, hybrids between p21 (152-159) and LDL motif (derived from E2F family transcription factors) or LFG motif (derived from p21 N-terminus, p27 and p57 kinase inhibitors), between p21 (16 – 23) and LIF motif (derived from p21 C-terminus) and between p21 (152-159)A153 and LFG motif. Their ability to inhibit CDK2/Cyclin E, CDK2/Cyclin A or CDK4/Cyclin D1 phosphorylation of pRb was compared with the one of the original peptides derived form p21- N and C-terminus, p27, E2F1 and p107 (Table 5).

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Table 5 Kinase inhibitory activity of LDL, LIF and LFG containing peptides, derived from E2F, p107, p21 N- and C-terminus and p27.

Peptide	Sequence	CDK 2-A IC50 [μM]	% Inhibition	CDK2-E IC50 [μM]	% Inhibition	CDK4-D1 IC50 [μM]	% Inhibition	CDK6-D1 IC50 [μM]	% Inhibition
P21 C-terminus	HSKRRLIF	3.4	80	3.4	80	21	72		
P21 C-term (S153A)	HAKRRLIF	0.021	88	0.35	81	6	82	5.8	100
P21 C-term ***LFG***	HSKRRLFG	1.4	78	1.6	82	Na	42		
P21 C-term LDL	HSKRRLDL	5.4	78	39	74	Na	24		
P21 C-term LFG(S153A)	HAKRRLFG	0.67	78	0.9	82	30	70		
E2F1	PVKRRLDL	1.2	80	2.1	74	99	58		
P27	SAURNLFG	6.1	80	2	82	Na	46		
P107	SAKRRLFG	0.73	75	0.5	86	17	78		
P21 N-term	KAURRLFG	0.54	80	0.34	86	42	66		
P21 N-term LIF	KAURRLIF	0.062	70	0.45	78	13	83		

5

The main results are:

1. All peptides inhibited CDK2-Cyclin and were much less potent toward CDK4/Cyclin

D1 kinase activity.

2. CDK2/Cyclin A and CDK2/Cyclin E were inhibited with similar potency by the 8-mers with the exception of HAKRRLIF and KAURRLIF which were 10 fold more potent toward CDK2/Cyclin A than to CDK2/Cyclin E kinase activity.
3. In the context of eight amino acid peptides alanine substitution of Ser153 led to significant increase of the kinase inhibitory potency of p21 (152-159) – 100, 10 and 4 fold toward CDK2/Cyclin A, CDK2/Cyclin E and CDK4/Cyclin D1 phosphorylation of pRb respectively.
4. The most potent inhibitors of pRb phosphorylation contain Ala on the second position and LIF motif; they are followed by the peptides containing Ala on the second position and LFG motif (with the exception of the p27 derived peptide which contain Gln instead of Arg on the 5th position), Ser and LFG, and Ser and LIF containing peptides. The least potent were LDL containing peptides.

These results manifest the importance of Ala and LIF motif for the kinase inhibitory potency of the peptides.

Competitive binding of peptides, containing different motifs (LIF, LFG, LDL) to Cyclin A or Cyclin D1.

The next important question was if these peptides share the same kinase inhibitory mechanism (bind to the same Cyclin docking site). To answer this question we developed a competitive binding assay where the influence of the 8-mers on Cyclin A (D1) – p21 (149-159) binding was studied (See Materials and Methods for more details).

The results from Cyclin D1 competitive binding assay are summarized on Table 6. For easy comparison, the data for CDK4/Cyclin D1 kinase inhibitory activity of the peptides are given in the same table.

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fold more

Table 6. Competitive binding of peptides, containing different motifs (LIF, LFG, LDL) to Cyclin D1.

Peptide	Sequence	CDK4-D1 IC50 [μM]	% Inhibition	Cyclin D1 competitive binding IC50 [μM]
P21 C-terminus	HSKRRLIF	21	72	48
P21 C-term (S153A)	HAKRRLIF	6	82	13
P21 C-term LFG	HSKRRLFG	Na	42	Non competitive
P21 C-term LDL	HSKRRLDL	Na	24	Non competitive
P21 C-term LFG(S153A)	HAKRRLFG	30	70	32.5
E2F1	PVKRRLDL	99	58	Non competitive
P27	SAURNLFG	Na	46	Non competitive
P107	SAKRRLFG	17	78	24.4
P21 N-term	KAURRLFG	42	66	134
P21 N-term LIF	KAURRLIF	13	83	19.8

We have demonstrated a very good agreement between the CDK4/Cyclin D1 kinase inhibition and Cyclin D1 competitive binding capabilities of the tested peptides. The highest potency to inhibit CDK4/Cyclin D1 phosphorylation of pRb and to compete with Biotinylated p21 – (149-159) for binding to Cyclin D1 has HAKRRLIF peptide. These results suggest a mode of kinase inhibition via binding to the cyclin and coincide well

th our previous results from 'pull down' experiments showing that the p21 (C-terminus) peptides bind to the Cyclins but not to the CDKs.

Thus, peptides containing the LDL motif (HSKRRLDL and PVKRRLDL) were not able to inhibit CDK4/Cyclin D1 or to compete with Biotin-DFYHSKRRLIF for binding to Cyclin D1. However, peptides, containing LFG motif and Ala on second position were able to inhibit CDK4/Cyclin D1 and to compete with Biotin-DFYHSKRRLIF for binding to Cyclin D1. The only exception of this rule is p27 derived peptide – SAURNLFG, where one of the important Arg is replaced with Asn. These results suggest that LFG and LIF peptides bind to the same site of Cyclin D1.

The results for Cyclin A competitive binding and CDK2/Cyclin A kinase inhibition of the peptides, containing LIF, LFG and LDL motifs are shown on Table 7. There is a very good correlation between the CDK2/Cyclin A inhibition and Cyclin A binding capabilities of the tested peptides. The most potent inhibitor and strongest binding competitor was HAKRRLIF peptide.

Table 7. Competitive binding of peptides, containing different motifs (LIF, LFG, and LDL) to Cyclin A .

Peptide	Sequence	CDK2-A IC50 [μM]	% Inhibition	Cyclin A competitive binding IC50 [μM]
P21 C-terminus	HSKRRLIF	3.4	80	nd
P21 C-term (S153A)	HAKRRLIF	0.021	88	0.3
P21 C-term LFG	HSKRRLFG	1.4	78	4.4
P21 C-term LDL	HSKRRLDL	5.4	78	5.8

P21 C-term LFG(S153A)	HAKRRLFG	0.67	78	0.35
E2F1	PVKRRLDL	1.2	80	1.2
P27	SAURNLFG	6.1	80	3.8
P107	SAKRRLFG	0.73	75	0.51
P21 N-term	KAURRLFG	0.54	80	0.75
P21 N-term LIF	KAURRLIF	0.062	70	0.3

nd – not determined

Specificity and selectivity of HAKRRLIF kinase inhibitory activity.

Similarly to p21 (149-160) its derivative p21 (152-153)153A was not able to inhibit Histone phosphorylation by CDK2/Cyclin A(E) complexes (data not shown). HAKRRLIF was not effective as an inhibitor in CDK1/Cyclin B in vitro kinase assay with Histone or Rb as substrates. HAKRRLIF did not inhibit PKCα induced phosphorylation of Histones.

Thus, we have defined a 8- amino acid peptide derived for p21 (C-terminus) with a single point mutation – S153A which has significantly higher kinase inhibitory activity than the original sequence. HAKRRLIF inhibited most strongly CDK2/Cyclin A phosphorylation of pRb – with IC 50 of 20 nM. The inhibitory activity of the peptide correlates with its ability to bind the cyclin sub-unit. HAKRRLIF is very selective and specific kinase inhibitor – it inhibits specificly only the pRb phosphorylation activity of G1 CDK/Cyclins and does not inhibit the mitotic CDK/Cyclins – CDK1/Cyclin B (or A), or PKC α. HAKRRLIF has much higher specificity and selectivity than the full length p21 protein, which inhibits the Histone phosphorylation of CDK2/Cyclin kinases complexes and has some activity toward CDK1/Cyclin B.

Example of a cyclin affinity capture method ofor the identification of peptide inhibitors

Peptides were synthesized as described above. Cyclin D1 was expressed in E coli BL21(DE3) using PET expression vector and purified from the inclusion bodies. After refolding Cyclin D1 was cross-linked on SulfoLink agarose support (PIERCE). CDK4-6 x His was expressed in Sf9 insect cells infected with the appropriate baculovirus construct and purified by metal-affinity chromatography (Quiagen). GST-Rb (773-924) was expressed in E coli and purified on a Glutathione-Sepharose column according the manufacturers instructions (Pharmacia). CDK4/Cyclin D1 phosphorilation of Rb was determined by incorporation of radio-labeled phosphate in GST-Rb in 96-well format kinase assay. The phosphorylation reaction mixture consisted of 50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, 20 mM -glicerophosphate, 2 mM NaF, 1 mM Na_3VO_4, 0.5 g CDK4, 0.5 g Cyclin D1, 10 l GST-Rb Sepharose beads, 100 M ATP and 0.2 Ci ^{32}P-ATP. The reaction was carried out for 30 min at 30 C at constant shaking. The GST-Rb-Sepharose beads were washed with 50 mM HEPES and 1 mM ATP and the radioactivity was measured on Scintillation counter (Topcount, HP)

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CLAIMS

1. A peptide of formula;

DFYHSKRRLIF (SEQ ID No. 1)

or such a peptide

(i) bearing a further amino acid residue at either end; or,

(ii) having upto 5 amino acid residues deleted from the N-terminal end;

and variants thereof wherein at least one amino acid residue is replaced by an alternative natural or unnatural replacement amino acid residue, with the proviso that the motif RXLXF is retained.

2. A peptide according to claim 1, wherein the further amino acid residue is selected from the polar residues C, N, Q, S, T and Y.

3. A peptide according to claim 2, wherein the amino acid residue is added to the N-terminal end.

4. A peptide according to claim 3, wherein the amino acid residue added is threonine.

5. A peptide according to claim 2, wherein the amino acid residue is added to the C-terminal end.

6. A peptide according to claim 5, wherein the amino acid residue added is serine.

7. A peptide according to claim 1, wherein upto 5 amino acid residues are deleted from the N-terminal end of SEQ ID No. 1.

8. A peptide according to claim 7, wherein from 2-4 amino acid residues are deleted from the N-terminal end of SEQ ID No. 1.

9. A peptide according to claim 8, wherein 3 amino acid residues are deleted from the N-terminal end of SEQ ID No. 1.

10. A peptide according to any of claims 7 to 9, wherein a further amino acid residue is added to the C-terminal end of the peptide.

11. A peptide according to claim 10, wherein the amino acid residue added is serine.

12. A variant according to claim 1, wherein the peptide of SEQ ID No 1 is modified by at least one of; deletion, addition or substitution of one or more amino acid residues, or by substitution of one or more natural amino acid residues by the corresponding D-stereomer or by a non-natural amino acid residue, chemical derivatives of the peptides, cyclic peptides derived from the peptides or from the peptide derivatives, dual peptides, multimers of the peptides and any of said peptides in the D-stereomer form, or the order of the final two residues at the C-terminal end are reversed.

13. A variant according to any preceding claim 1, wherein the serine residue corresponding to p21(153Ser), is replaced by an alanine residue.

14. A peptide according to any preceding claim, selected from;

DFYHSKRRLIFS
TDFYHSKRRLIF,
AFYHSKRRLIFS,
DAYHSKRRLIFS,
DFAHSKRRLIFS,
DFYASKRRLIFS,
DFYHAKRRLIFS,
DFYHSARRLIFS,
DFYHSKRALIFS,
DFYHSKRRLAFS,
DFYHSKRRLIFA,
FYHSKRRLIFS,

YHSKRRLIFS,
HSKRRLIFS,
DFYHSKRRLIF,
FYHSKRRLIF
YHSKRRLIF
HSKRRLIF,
SKRRLIF,
KRRLIF.

15. A peptide of formula (I);

$$X_1X_2X_3RX_4LX_5F \qquad (I)$$

wherein X_1, X_3, X_4 and X_5 may be any amino acid and X_2 is serine or alanine; and variants thereof with the proviso that the motif RX_4LX_5F is retained.

16. A peptide according to claim 15, wherein X_5 is selected from isoleucine and glycine.

17. A peptide according to claim 15 or 16, wherein X_1 and X_4 are both basic amino acid residues, X_1 is more preferably histidine and X_4 more preferably arginine. X_3 is a basic or polar residue,.

18. A peptide according to claim 17, wherein X_1 is histidine and X_4 is arginine, and X_3 is lysine or cysteine.

19. A peptide according to claim 15 of the formula;

$$HX_2KRRLX_5F \text{ (SEQ ID No. 2)}$$

20. A peptide according to any of claims 15 to 19, wherein X_2 is alanine.

21. A peptide according to claim 20, wherein X_5 isoleucine.

22. A variant according to any of claims 15 to 21, wherein the peptide is modified by at least one of; deletion, addition or substitution of one or more amino acid residues, or by substitution of one or more natural amino acid residues by the corresponding D-stereomer or by a non-natural amino acid residue, chemical derivatives of the peptides, cyclic peptides derived from the peptides or from the peptide derivatives, dual peptides, multimers of the peptides and any of said peptides in the D-stereomer form, or the order of the final two residues at the C-terminal end are reversed.

23. A variant according to claim 22, wherein the order of X_5 and F are reversed.

24. A peptide according to any of claims 15 to 23, selected from;

HSKRRLIF,
HAKRRLIF,
HSKRRLFG,
HAKRRLFG,
KACRRLFG,
KACRRLIF.

25. An assay for identifying candidate substances capable of binding to a cyclin associated with a G1 control cdk enzyme and/or inhibition of said enzyme, comprising;
(a) bringing into contact a peptide as defined in any of claims 1-22, said cyclin, said cdk and said candidate substance, under conditions wherein, in the absence of the candidate substance being an inhibitor of interaction of the cyclin/cdk intteraction, the p21 derived peptide would bind to said cyclin, and
(b) monitoring any change in the expected binding of the p21 derived peptide and the cyclin.

26. An assay according to claim 25, wherein at least one of the assay components is bound to a solid phase.

27. An assay according to claim 26, wherein the peptide is labelled such as to emit a signal when bound to said cyclin.

28. An assay according to claim 26, wherein the cyclin is labelled such as to emit a signal when bound to the peptide.

29. An assay according to any of claims 25 to 28, wherein the cyclin is cyclin A or E and the cdk is cdk2.

ABSTRACT

The present invention relates to p21 derived peptides capable of inhibiting cdk/cyclin complexes, particularly cyclins A or E/cdk2, by modifying the interaction with their substrates. The peptides are derived from a C-terminal region of p21 and display selectivity for cyclin/cdk2 inhibition over cyclin/cdk4 inhibition. Variants of such peptides particularly involving certain alanine replacements are shown to be particularly potent.

igure 1. Effect of p21 (149-160) on CDK2-Cyclin E induced phopshorylation of different concentrations Histone 1. Yellow line (+)– 1mg/ml Histone1, purple line (diamonds), 0.7 mg/ml Histone 1, blue line (x)– 0.25 mg/ml Histone 1 and brown line (closed circles) – 0.1 mg/ml Histone 1.

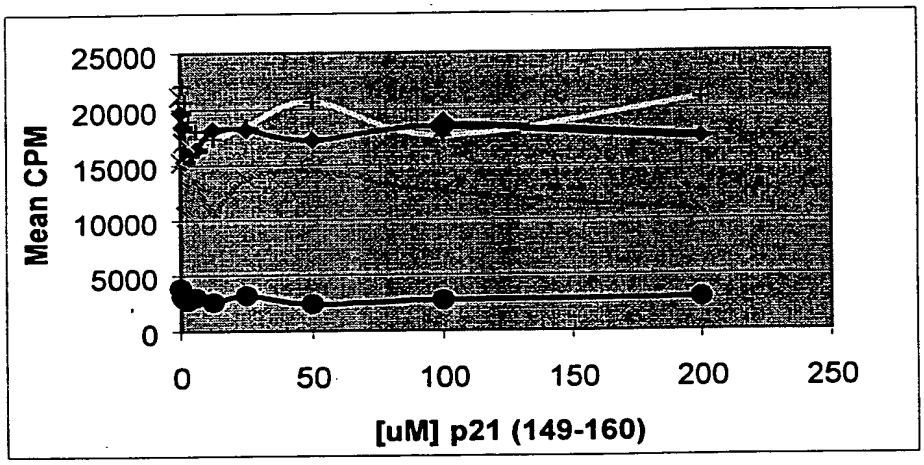
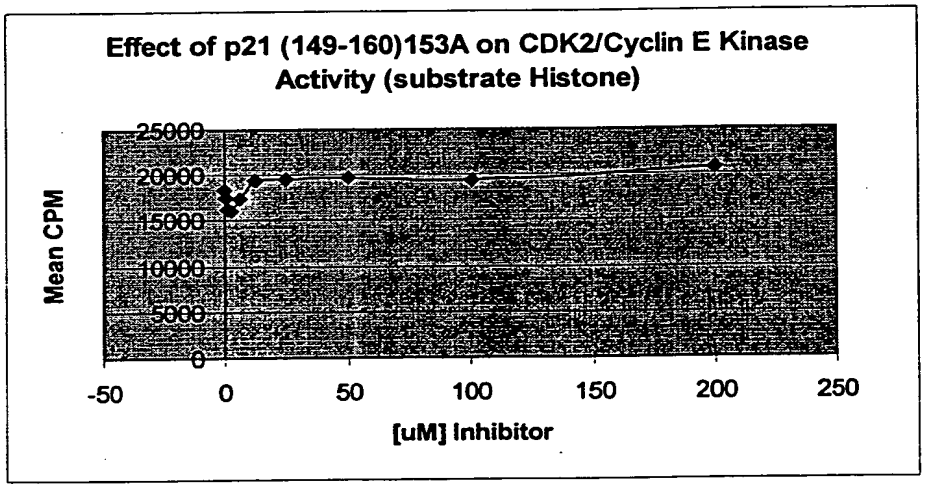


Figure 2. p21 (141-160)153A is a strong inhibitor of GST-Rb phopshorylation but not of Histone 1 phosphorylation induced by CDK2-Cyclin E kinase complex.



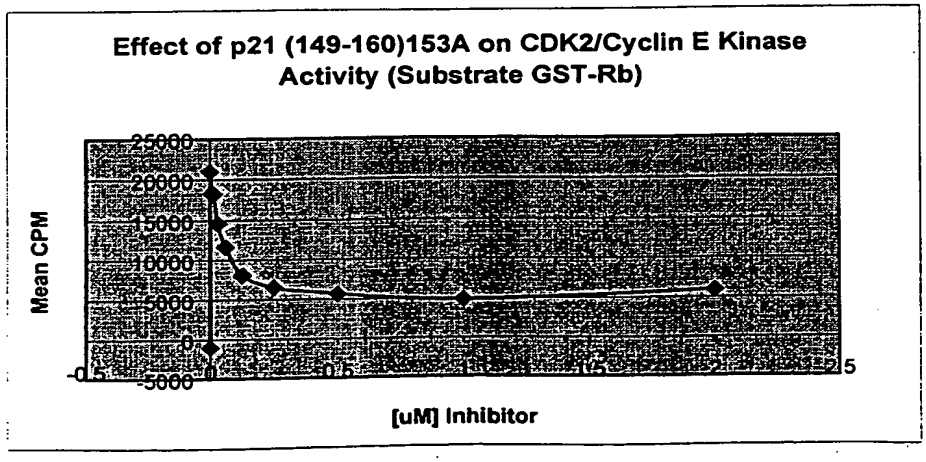


Figure 3. Binding of biotinylated – p21 (149-159) to CDK2, CDK4, Cyclin D1, or Cyclin A when they are monomers or dimeric complexes. In the first column is shown the antibody used for the immunodetection; in the second – the profiles of the "pulled down" proteins; the third – starting material for the binding reaction.

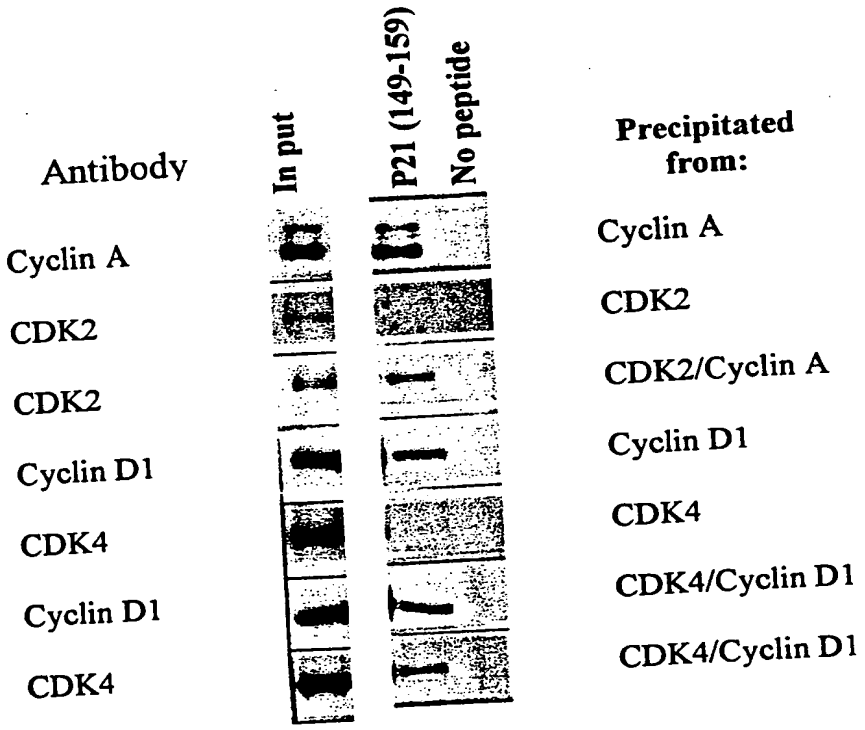


FIGURE 4.

	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	***his***	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	***ala***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	***lys***	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	***arg***	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	***arg***	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***leu***	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	***ile***	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***phe***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	***Ala***	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
Ala/Gly scan	H-	His	***Gly***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	***Ala***	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	***Ala***	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	***Ala***	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***Ala***	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	***Ala***	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	-	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
Ile7 deletion	H-	***Pya***	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
His1 replacements	H-	***Thi***	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	***Hse***	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	***Phe***	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_3
	H-	***Dab***	Ala	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	***Abu***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
Ala2 replacements	H-	His	***Nva***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	***Bug***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	***Val***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	***Ile***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	***Phg***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	***Phe***	Lys	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	***Nle***	Arg	Arg	Leu	Ile	Phe	-NH_2
Lys3 replacements	H-	His	Ala	***Abu***	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	***Leu***	Arg	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	***Cit***	Arg	Leu	Ile	Phe	-NH_2
Arg4 replacements	H-	His	Ala	Lys	***Hse***	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	***His***	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	***Nle***	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	***Gln***	Arg	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	***Asn***	Leu	Ile	Phe	-NH_2
Arg5 replacements	H-	His	Ala	Lys	Arg	***Pro***	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	***Ser***	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	***Aib***	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	***Sar***	Leu	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***leu***	Ile	Phe	-NH_2
Leu6 replacements	H-	His	Ala	Lys	Arg	Arg	***Ile***	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***Nle***	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***Nva***	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***Cha***	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***Phe***	Ile	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	***1Nap***	Leu	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	***Leu***	Phe	-NH_2
Ile7replacements	H-	His	Ala	Lys	Arg	Arg	Leu	***Nle***	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	***Nva***	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	***Cha***	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	***Phe***	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	***1Nap***	Phe	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***Leu***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***Cha***	-NH_2
Phe8 replacements	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***Hof***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***Tyr***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***pFPhe***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***mFPhe***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***Trp***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***1Nal***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***2Nal***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***Bip***	-NH_2
	H-	His	Ala	Lys	Arg	Arg	Leu	Ile	***Tic***	-NH_2